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Research Article

Multi-locus environmental DNA markers and taxon-specific primers enhanced detection rate of invasive tilapia

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Abstract

Tilapia are native to Africa. Due to introductions for aquaculture and the ornamental fish trade, several species of tilapia have now spread across five continents, making them some of the most ecologically harmful invasive species. During the early stages of their escape, tilapia are often difficult to detect using traditional fish survey methods. Using environmental DNA (eDNA) methods to monitor tilapia invasion is a promising supplementary approach, but currently no eDNA markers or suitable primers have been developed specifically for tilapia. In this study, we designed four pairs of tilapia-specific primers for use in multiplex PCR coupled with high-throughput sequencing, enabling the simultaneous detection of multiple tilapia species in a single reaction. We found that the taxon-specific primers (Tila-1–4) exhibited higher detection sensitivity compared to the universal primer (tele02), reaching 5.33×10^{-7} ng/μL. Furthermore, multi-locus amplification reached a 95% limit of detection (LOD) at 2.66×10^{-6} ng/μL, which was significantly better than 2.3×10^{-3} ng/μL for single-locus amplification. Finally, we collected water samples from nine rivers across four drainages in China to test the method that we developed. The results indicated that tilapia have invaded river systems such as the Yangtze River and the Pearl River. Specifically, *Oreochromis niloticus* and *Coptodon zillii* were the dominant invasive species detected, with some locations also showing the presence of *O. aureus* and *Sarotherodon galilaeus*. This method provides an effective tool for early monitoring of tilapia invasion, which may inspire the development of similar strategies for monitoring other invasive species.

Key words: Environmental DNA, invasive species, multiplex PCR, species monitoring, taxon-specific primers, tilapia

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Introduction

Biological invasions are a threat to biodiversity worldwide. With the acceleration of economic globalization, the rate of introduction of invasive species has rapidly increased, and the resulting damage has become more severe (Simberloff et al. 2013). Compared to terrestrial ecosystems, aquatic ecosystems are more susceptible to invasions by non-native species (Strayer 2010). Invasive fish species are a typical example, as they threaten native biodiversity through predation, hybridization, and competition, disrupting habitats and ecosystem functions (Ottinger et al. 2016).

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Since the introduction and spread of aquatic invasive species pose a major threat to aquatic ecosystems, early detection of these invaders is crucial (LeBlanc et al. 2020).

Traditional monitoring techniques, such as those based on catch and fishery surveys, face challenges due to increasing fishing bans in many areas, highlighting the urgent need for alternative monitoring methods. Environmental DNA (eDNA) is a method of extracting and analyzing genetic material from environmental samples, which contain traces of various organisms present in that environment. By analyzing these DNA samples, species occurrence and abundance can be inferred (Thomsen and Willerslev 2015). Compared to traditional monitoring methods, eDNA offers higher sensitivity, greater efficiency, and non-invasiveness, making it ideal for detecting rare or elusive species. This approach has proven particularly successful for finfish detection, as fish continuously shed DNA into the aquatic environment through mucus, scales, feces, gametes, and sloughed epithelial cells (Nagarajan et al. 2022). These processes contribute to relatively high concentrations of extracellular DNA in water, increasing the likelihood of detection. Universal primers targeting the mitochondrial 12S region, such as the MiFish set, amplify a 170 bp fragment and are widely used in fish eDNA metabarcoding, consistently showing high amplification efficiency (Miya et al. 2015). The universal 12S primers tele02 (Taberlet et al. 2018), which amplify a 167 bp fragment, are also commonly used for metabarcoding fish diversity. Other studies have shown that taxon-specific primers often yield better results when detecting individual species than universal primers (Thalinger et al. 2023). Additionally, amplifying multiple loci of the target species can significantly enhance detection sensitivity (Brys et al. 2023).

Tilapia belong to the order Cichliformes and the family Cichlidae, which includes hundreds of species across three genera: *Tilapia*, *Sarotherodon*, and *Oreochromis*. Tilapia are characterized by strong environmental adaptability and tolerance to low oxygen levels and a wide range of salinity (Elsaied et al. 2019). Most tilapia are warm-water fishes with an optimal survival temperature of 28–32 °C and a critical survival temperature of 10–41 °C (Platt and Hauser 1978). When water temperatures drop below 15 °C, tilapia stop feeding completely, and they are likely to die when temperatures fall below 10 °C (Gu et al. 2018). However, *Coptodon zillii* has a strong cold tolerance and can survive at temperatures as low as 6.5 °C (Yue et al. 2024). Today, they play a key role in small- and large-scale aquaculture, often increasing farmers' income and mitigating poverty. However, with their escape from aquaculture and subsequent spread, tilapia have successfully invaded inland waters across southern China and have become naturalized in some regions as common species (Kang et al. 2023).

In this study, we aimed to develop novel taxon-specific markers within a metabarcoding framework, using multiplex PCR and high-throughput sequencing, to enable the simultaneous detection of multiple tilapia species from environmental DNA samples. Specifically, our objectives were to (1) compare the detection sensitivity of tilapia-specific primers with that of universal fish eDNA primers; (2) assess the performance of single-locus versus multi-locus amplification within the context of metabarcoding; and (3) select and optimize the best primer combinations and PCR conditions for detecting tilapia in eDNA samples. To field test our new primers, we collected water samples from nine rivers across four drainage systems in China (the Huai–Yangtze River Drainage, the Qiantang River Drainage, the Minjiang River Drainage, and the Pearl River Drainage) and used our optimized method to estimate tilapia invasion status in these waters.

Materials and methods

To provide a clear overview of the experimental design, we included a workflow diagram summarizing the major steps of the study (Fig. 1).

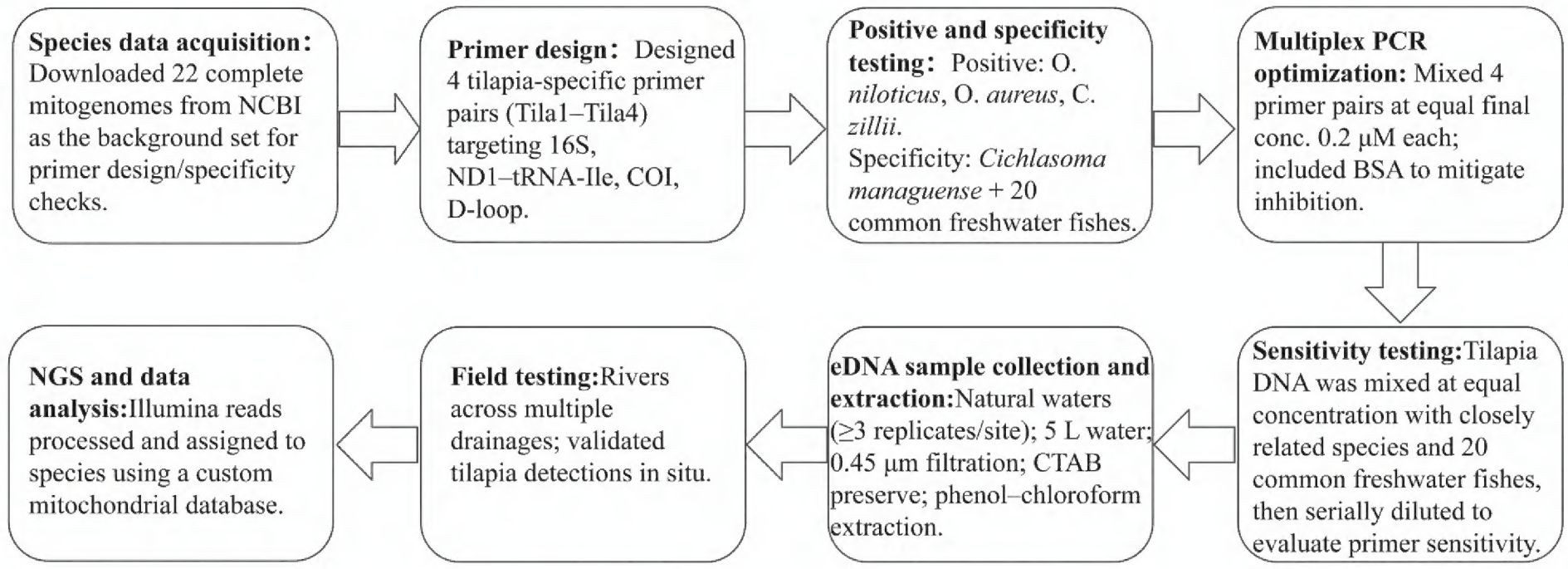
Species data acquisition: Downloaded 22 complete mitogenomes from NCBI as the background set for primer design/specificity checks.
Primer design: Designed 4 tilapia-specific primer pairs (Tila1–Tila4) targeting 16S, ND1–tRNA-Ile, COI, D-loop.
Positive and specificity testing: Positive: O. niloticus, O. aureus, C. zillii. Specificity: Cichlasoma managuense + 20 common freshwater fishes.
Multiplex PCR optimization: Mixed 4 primer pairs at equal final conc. 0.2 µM each; included BSA to mitigate inhibition.
Sensitivity testing: Tilapia DNA was mixed at equal concentration with closely related species and 20 common freshwater fishes, then serially diluted to evaluate primer sensitivity.
eDNA sample collection and extraction: Natural waters (≥3 replicates/site); 5 L water; 0.45 µm filtration; CTAB preserve; phenol–chloroform extraction.
Field testing: Rivers across multiple drainages; validated tilapia detections in situ.
NGS and data analysis: Illumina reads processed and assigned to species using a custom mitochondrial database.

Figure 1. Workflow of the experimental design.

Design of tilapia-specific primers targeting different loci

To design primers for the specific amplification and detection of target tilapia fragments, two requirements were considered: (1) the primers must amplify only tilapia species, without amplifying non-target cichlids or other fish species found in the same region; and (2) the amplified fragments must contain sufficient sequence variation to distinguish among different tilapia species. Current primer design software generally designs primers based on a single sequence or does not consider variations in the amplified sequences, making it unable to meet these two requirements. Therefore, we adopted a manual design approach based on experience, supplemented by software validation, to develop markers and design primers. Briefly, the complete mitochondrial genomes of several species were downloaded from the NCBI public database (https://www.ncbi.nlm.nih.gov/), including *Atherinomorus stipes* (NC_063115.1), *Cheilopogon spilonotopterus* (NC_039400.1), *Plesiops nakaharae* (NC_034341.1), *Ambassis gymnocephalus* (NC_033497.1), *Amphiprion sebae* (NC_030591.1), *Embiotoca jacksoni* (NC_029362.1), *Melanotaenia splendida* (NC_028280.1), *Oedalechilus labiosus* (NC_025941.1), *Opistognathus jacksoniensis* (NC_017895.1), *Oryzias celebensis* (NC_049128.1), *Abudefduf bengalensis* (NC_041192.1), *Chirostoma humboldtianum* (NC_024883.1), *Atherinopsis californiensis* (NC_067733.1), *Iriatherina werneri* (NC_036767.1), *Omobranchus fasciolatoceps* (NC_060850.1), *Hyporhamphus paucirastris* (NC_065259.1), *Parachromis managuensis* (NC_026918.1), *Oreochromis mossambicus* (NC_007231.1), *Oreochromis aureus* (NC_013750.1), *Oreochromis niloticus* (NC_013663.1), *Coptodon zillii* (NC_026110.1), and *Sarotherodon galilaeus* (MW_194078.1). These 22 species were selected to represent a broad phylogenetic range across teleost fishes, including species from families such as Atherinidae, Belonidae, Blenniidae, Cichlidae, and Poeciliidae. Several of these species are also known to occur in the same habitats as invasive tilapia in China or are closely related taxa, making them suitable background references for assessing primer specificity. The downloaded gene

sequences were aligned using MEGA11 software. Based on the aligned sequences, primers were manually designed to ensure that they specifically amplify tilapia sequences and not those of other background species. Specificity requirements for primer design were as follows: the target species and all background species should have at least 3 bp differences in the primer binding region and should ideally meet at least one of the following conditions: (1) the last base at the 3' end is different; (2) 2 of the last 4 bases at the 3' end are different; or (3) 3 of the last 6 bases at the 3' end are different. The designed primers were then checked for common issues, such as hairpin structures, using the Primer3 web tool (https://primer3.ut.ee/). The screening parameters were as follows: amplicon size, 100–150 bp; primer length, 22–29 bp; annealing temperature, 53–57 °C; GC content, 20–80; hairpin < 40; polyX < 7. Other parameters were set according to the default settings of Primer3.

Primer validation (first-stage PCR)

To evaluate the performance of the newly designed primers, we first confirmed the mitochondrial regions targeted by each primer set. Tila1–Tila4 target the 16S, ND1-tRNA-Ile, COI, and D-loop regions, respectively (Table 1; Suppl. material 1), all of which are part of the multicopy mitochondrial genome. Although many species of tilapia have been introduced into China, the most common ones are *Oreochromis niloticus*, *Oreochromis aureus*, and *Coptodon zillii*. Therefore, we selected these three species for testing our new primers. A two-stage PCR protocol was employed for library construction. The first-stage PCR (PCR1) consisted of two parts: (I) single-primer assays and (II) multiplex PCR assays. During single-primer assays, two tests were conducted: (1) positive tests – each of the three target tilapia species was amplified in separate PCR reactions to confirm primer efficacy; and (2) specificity tests – DNA from the non-tilapia cichlid *Cichlasoma managuense* was amplified for each primer set, and DNA from 20 common freshwater fish species was pooled at equal concentrations and amplified in single PCR reactions. The 20 common freshwater fish species included *Channa argus*, *Paramisgurnus dabryanus*, *Hypophthalmichthys molitrix*, *Xenocypris argentea*, *Hypophthalmichthys nobilis*, *Culter alburnus*, *Rhodeus ocellatus*, *Cyprinus carpio*, *Paracanthobrama guichenoti*, *Tachysurus fulvidraco*, *Saurogobio dumerili*, *Pseudorasbora parva*, *Abbottina rivularis*, *Micropercops swinhonis*, *Carassius auratus*, *Hemibarbus maculatus*, *Ctenopharyngodon idellus*, *Hemiculter leucisculus*, and *Mylopharyngodon piceus*. These species are commonly found in natural freshwater bodies across China, representing typical

Table 1. Four primer pairs amplifying mitochondrial regions of tilapia, including primer sequences and targeted regions.

Primer set	Primer sequences (5'-3')	Start position	Ending position	Locus	Amplicon Length (bp)
Tila1	AACGAACCATTCACCGACCATTAAC	1844	1985	16S	142
	CTTTTTCTTTTAGTCTTTCCCGGTTGG				
Tila2	CCCCAGAAGTGACAACAACAAACC	3628	3859	ND1-tRNA-Ile	232
	CCCTTTATTTCAGGCACAACTCC				
Tila3	CCTGCCTTCGTCCAAGTTCAA	6999	7091	COI	93
	CAATTCCTCCCTTTCTCGCTAGTG				
Tila4	GAATTGCATAACTGATATCAAGAGCATAA	16336	16485	D-loop	150
	GGGGGGTTTACAGGAGTATTAGAGA				

non-target taxa in tilapia-invaded habitats. The PCR reaction mixture consisted of 5 μL of Takara enzyme mix (Takara Bio, Japan), 0.4 μL of each forward and reverse primer (400 nM final concentration), 0.8 μL of DNA template, and ddH2O to a final volume of 10 μL. PCR conditions for PCR1 were as follows: initial denaturation at 95 °C for 5 min, followed by 16 cycles of denaturation at 95 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 45 s, followed by 16 cycles at 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 45 s, with a final extension at 72 °C for 5 min, and holding at 8 °C. One microliter of PCR products was subjected to 1.5% agarose gel electrophoresis. The gel was imaged to observe the bright bands of target fragments, which were used to determine the presence of tilapia. Negative controls were included in each PCR run.

Optimization of reaction conditions of the multiplex PCR

Based on the successful results of the individual primer tests, we combined four primer pairs in multiplex PCR reactions, with each pair mixed at an equal final concentration of 0.2 μM. These were tested on DNA extracted from tilapia tissue samples, water samples from a tilapia aquaculture tank, and natural water samples. The tissue samples included single or multiple tilapia specimens; aquaculture water samples were collected from cement ponds of a tilapia farm; and natural water samples were directly collected from the wild where tilapia were observed (Fig. 2).

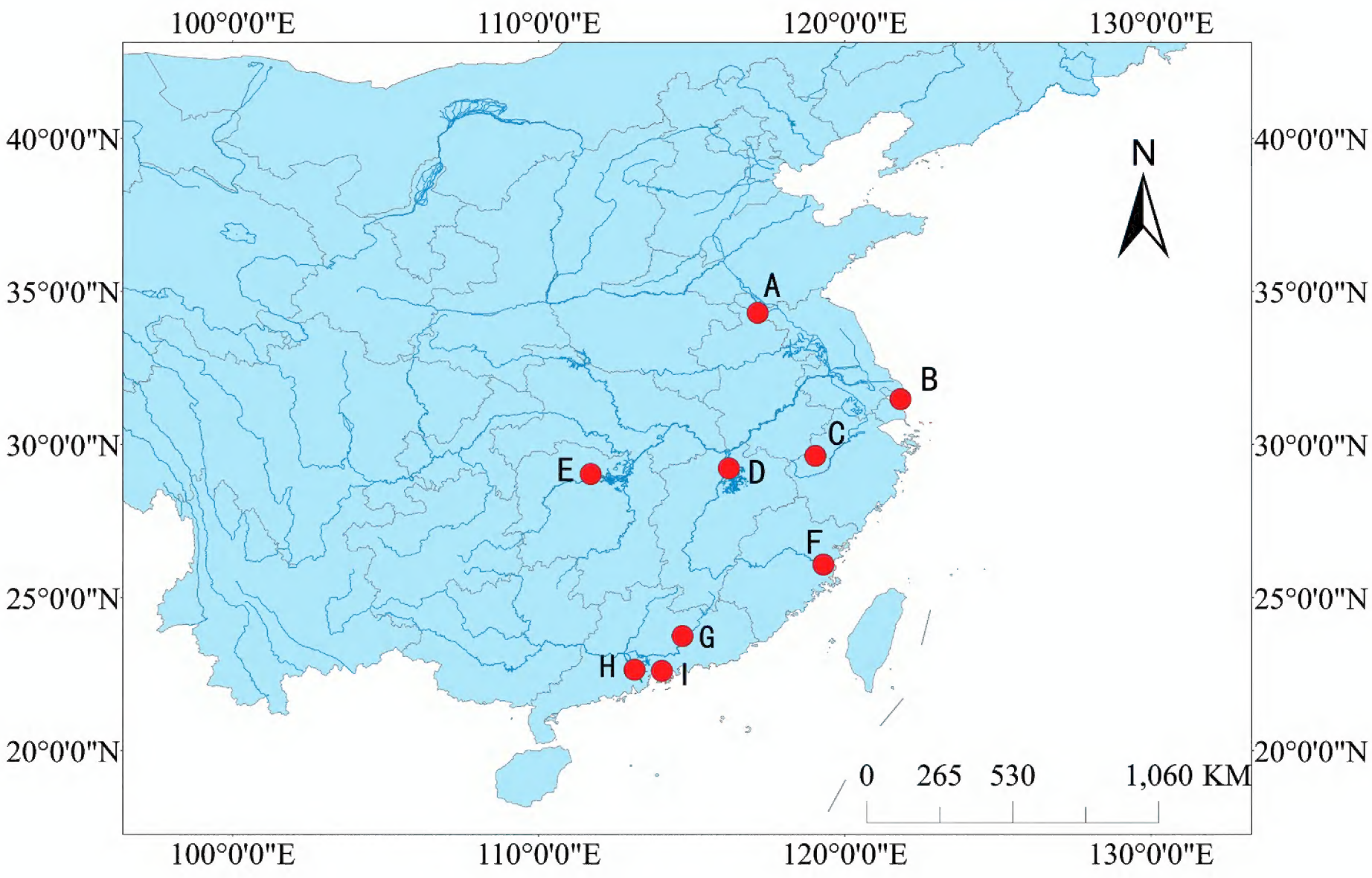

Figure 2. Sampling sites for field water sample testing. **A.** The Yihe River (the Huai River Basin); **B.** The Yangtze River; **C.** The Xin'an River (the Qiantang River Basin); **D.** The Ganjiang River (the Yangtze River Basin); **E.** The Yuan River (the Yangtze River Basin); **F.** The Minjiang River; **G.** The Dongjiang River (the Pearl River Basin); **H.** The Xijiang River (the Pearl River Basin); **I.** The Shenzhenhe River (the Pearl River Basin).

Methods for water sample collection, filtration, and DNA extraction are detailed in the section "Field testing of the optimized protocol". BSA was added as an anti-inhibition agent to all PCR reactions to alleviate potential PCR inhibition in environmental samples (Deiner et al. 2015; Jane et al. 2015). We optimized a new PCR1 condition for multiplex reactions: 5 μL of 2× Multiplex PCR Master Mix enzyme (QIAGEN, Germany), 0.5 μL of BSA (Macklin, China), 3 μL of DNA template, 0.4 μL each of the four selected forward and reverse primers (10 μmol/L), and ddH2O to a final volume of 10 μL. The reaction conditions were as follows: initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 95 °C for 30 s, annealing at 56.6 °C for 90 s, and extension at 72 °C for 30 s; followed by a final extension at 68 °C for 10 min. The amplification success of multiplex PCR was evaluated by agarose gel electrophoresis, where the presence of expected bands indicated successful amplification.

Primer sensitivity test and comparison with universal fish eDNA primers, tele02

Genomic DNA of *C. zillii*, *O. niloticus*, and *O. aureus* was mixed with DNA of the 20 common freshwater fish and *C. managuense* at equal concentrations to simulate a mock natural environment sample. The tilapia DNA mixture (from *C. zillii*, *O. niloticus*, and *O. aureus*) was initially quantified at 5.21 ng/μL using a Nanodrop 3300 spectrophotometer (Thermo Fisher Scientific) and then serially diluted in 1:5 steps to produce 10 concentrations: 5.21 ng/μL, 1.04 ng/μL, 0.21 ng/μL, 0.04 ng/μL, 0.008 ng/μL, 0.002 ng/μL, 3×10^{-4} ng/μL, 6.66×10^{-5} ng/μL, 1.33×10^{-5} ng/μL, 2.66×10^{-6} ng/μL, and 5.33×10^{-7} ng/μL. These dilutions were prepared based on theoretical volume ratios without re-measuring DNA concentration after each step. The detection limit of each primer was measured using DNA samples with different concentrations of tilapia DNA. Detection limit tests were also conducted for primer combinations under the same conditions. Three replicate experiments were performed for each concentration. Universal primers targeting the mitochondrial 12S region are more effective for monitoring fish than the COI and Cytb regions and are, therefore, used more frequently (Zhang et al. 2020). For species-specific monitoring, eDNA barcoding has higher sensitivity than eDNA metabarcoding and universal primers (Xia et al. 2021). To verify this, we compared the tilapia-specific primers with the universal primers (tele02) to evaluate their performance in detecting low concentrations of the target tilapia species. All amplification results were subjected to next-generation sequencing. Amplification steps followed those described in the section "Optimization of reaction conditions for multiplex PCR", and detailed steps for library preparation, sequencing, and data analysis are provided in the section "Library construction, sequencing, and data analysis".

To evaluate detection sensitivity under low-DNA conditions, we conducted single-locus and multi-locus PCR reactions on a gradient of serially diluted tilapia DNA samples. For each dilution level, 20 technical replicates were tested to determine the minimum DNA concentration achieving a 95% detection probability, which we defined as the limit of detection (LOD). Following conventions in metabarcoding assays (McCarthy et al. 2023; Wichianchot et al. 2022; Tobar et al. 2025), we report LODs strictly in terms of DNA concentration.

Field testing of the optimized protocol

Based on tilapia's preference for warm temperatures and existing literature reports (Yongo et al. 2023), we selected sampling points in river systems south of the Huai–Yangtze River Drainage. Collecting multiple field replicates was necessary to improve the effectiveness of DNA capture and the likelihood of detecting tilapia eDNA (Ruppert et al. 2022). Notably, most studies collected at least three samples from the same site (Hinlo et al. 2018), so we ensured at least three replicate sampling points for each river (Fig. 2). Water samples were collected using a 5 L plexiglass water sampler according to the requirements of the Chinese National Standard HJ 91.2–2022, "Technical specifications for surface water environmental quality monitoring" (Ministry of Ecological Environment of the People's Republic of China 2022). DNA enrichment was performed using a positive-pressure filter with a 0.45 μm mixed cellulose ester filter membrane (PALL, Michigan, USA, 66191). The filter membrane was placed in CTAB (Solarbio, China) preservation solution and stored at –20 °C. Common DNA extraction methods include kit extraction and the phenol–chloroform–isopropanol method (Djurhuus et al. 2017). Since we found that many sampling sites contained high amounts of particulate matter, which may negatively affect DNA extraction when using kits, we chose the phenol–chloroform–isopropanol method, which allows particle sedimentation by centrifugation. To monitor potential contamination during field sampling, field blanks consisting of sterilized ddH_2O were exposed to the same handling procedures as the environmental water samples.

Library construction (second-stage PCR), sequencing, and data analysis

PCR products obtained from the first-stage PCR were subsequently amplified in a second-stage PCR (PCR2) to incorporate Illumina adapters and 8-bp dual-index barcodes, facilitating multiplexed sequencing. Specifically, 4 μL of each PCR1 product was amplified using standard Illumina P5 and P7 indexed primers. The reaction mixture was as follows: 10 μL of 2× Multiplex PCR Master Mix enzyme (QIAGEN, Germany), 4 μL of DNA template, 1 μL of P5/P7 primers (10 μmol/L), and nuclease-free water to a final volume of 20 μL. The reaction conditions were: 95 °C for 5 min for initial denaturation, followed by 15 cycles of 95 °C for 15 s for denaturation, 53 °C for 15 s for annealing, and 72 °C for 15 s for extension, with a final extension at 72 °C for 5 min. Amplified products were analyzed using 1.5% agarose gel electrophoresis, and samples with bands meeting sequencing requirements were subjected to high-throughput sequencing using the Illumina NovaSeq 6000 platform at Novogene (Beijing, China). Each sample was sequenced in paired-end 150 bp (PE150) mode, with an average of 1.6–1.7 million paired-end reads per sample. All sequencing data were subjected to bioinformatic analysis on a Linux server.

We used Fastp (version 0.12.4) for raw quality control, setting a quality threshold of 30 for base calls, a minimum length of 50 bp, and limiting the proportion of bases with low-quality scores to no more than 2% (Chen et al. 2018). Pandaseq (version 2.11) was then used with default parameters to merge forward and reverse sequencing reads (Masella et al. 2012). Samples were subsequently demultiplexed based on the unique primer sequences of each target locus using a Python script (https://github.com/enormandeau/barque), allowing up to two mismatches in the primer regions to account for sequencing errors. This step

enabled the bioinformatic separation of amplicons by locus (Tila1–Tila4) prior to downstream analysis so that reads from each marker were processed as independent datasets throughout the pipeline. All loci were processed under identical bioinformatic parameters. Finally, target sequences were filtered based on their amplified length, with only sequences falling within ±10% of the expected amplicon length being retained (Ratnasingham 2019).

To address potential issues with repeated amplification of the same template, which can lead to abundant duplicate sequences, VSEARCH (version 2.18.0) was used to filter out duplicate sequences. Sequences were then clustered into ASVs using the unoise algorithm (Rognes et al. 2016). To reduce false-positive detections from tag-jumping, sequencing noise, or low-level contamination, we applied a conservative threshold: a tilapia species was considered present only if it accounted for ≥0.1% of the total reads at that locus. This cutoff is supported by previous studies, which recommend thresholds ranging from 0.01% to 0.1% to minimize false positives (Deiner et al. 2017; Bista et al. 2017). Considering the complexity of environmental samples and our multiplexed design, 0.1% was chosen as a reliable and conservative value. Once ASV sequences were obtained, they were assigned to species using BLASTN (version 2.3.31) (Camacho et al. 2009). Taxonomic assignments were performed against a custom local reference database constructed by merging publicly available mitochondrial sequences from NCBI, the MitoFish database (Sato et al. 2018; Zhu et al. 2023), and datasets from the BOLD barcode database (Ratnasingham and Hebert 2007). The final database contained more than 30,000 sequences, ranging in length from short 12S rRNA fragments (~92 bp) to complete mitochondrial genomes (~18,000 bp), representing 3,236 genera and 11,236 fish species across both freshwater and marine taxa. A similarity threshold of ≥97% was applied for genus-level assignments, following common practice in fish eDNA metabarcoding studies (Bessey et al. 2020; Dal Pont et al. 2021; Sales et al. 2021). In practice, all matches were greater than 99%, with most reaching 100%.

Results

Selection of primer combinations

Seven primer pairs were designed to amplify partial fragments of the mitochondrial genes 16S rRNA, ND1, ND2, COI, and D-loop. Positive testing showed that two primer pairs designed to target the mitochondrial ND2 and D-loop regions did not amplify *C. zillii*, and another primer pair targeting the D-loop region failed to amplify *C. zillii*, *O. niloticus*, and *O. aureus*. Therefore, these three primer pairs were excluded from further testing. Ultimately, four primer pairs were selected for the multiplex PCR experiments, named Tila1, Tila2, Tila3, and Tila4 (Table 1).

Evaluation of primer specificity, sensitivity, and performance in multiplex PCR amplification

All four primer pairs did not amplify the 20 common freshwater fish species or the closely related cichlid species (Fig. 3A). The multiplex PCR reaction showed that amplification efficiency was highest in DNA extracted from tilapia tissue samples, followed by water samples collected from an aquaculture tank containing tilapia, and lowest in samples collected from natural water (Fig. 3B).

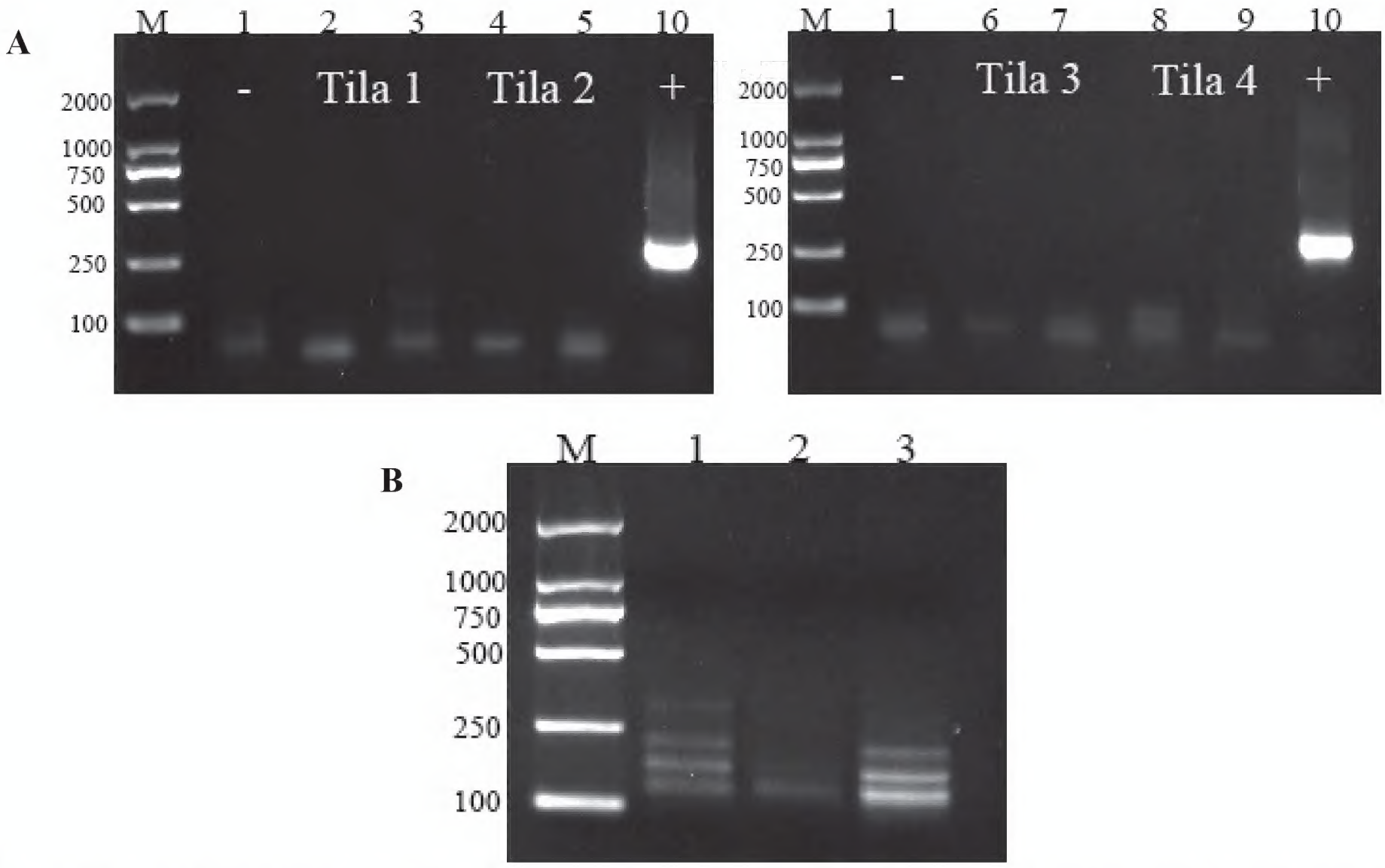

Figure 3. A. Specificity testing of the four tilapia-specific primers. M: marker; lane 1 is a blank control (primer: Tila 1), lanes 2–3 are Tila 1, lanes 4–5 are Tila 2, lanes 6–7 are Tila 3, lanes 8–9 are Tila 4, and lane 10 is a positive control using *O. niloticus* DNA (primer: tele02); paired lanes show the results of the 20 species mixture and *Cichlasoma managuense*. **B.** Multiplex PCR amplification using the mixed primer set (Tila1–Tila4). M: marker; lane 1: *O. niloticus* tissue DNA; lane 2: sample collected from natural water; lane 3: water sample collected from an aquaculture tank containing tilapia.

Table 2. Detection sensitivity (ng/μL) of each primer across three target tilapia species.

primer	*O. niloticus*	*C. zillii*	*O. aureus*
Tila 1	0.83×10^{-2}	5.33×10^{-7}	0.13×10^{-4}
Tila 2	5.33×10^{-7}	5.33×10^{-7}	0.13×10^{-4}
Tila 3	1.33×10^{-5}	5.33×10^{-7}	2.67×10^{-6}
Tila 4	0.83×10^{-2}	5.33×10^{-7}	0.21
Tele02	5.33×10^{-7}	0.04	0.21

The detection limits of each primer for the three tilapia species under single-primer assays are listed in Table 2. For *O. niloticus*, the specific primer Tila2 had similar sensitivity to the universal tele02, while Tila1, Tila3, and Tila4 had lower sensitivity than tele02. For *C. zillii*, all specific primers (Tila1, Tila2, Tila3, and Tila4) showed higher sensitivity than tele02. For *O. aureus*, specific primers Tila1, Tila2, and Tila3 showed higher sensitivity than tele02, while Tila4 had the same sensitivity as tele02. In most cases, the specific primers exhibited higher or equivalent sensitivity compared to the universal primer. These results confirm the earlier hypothesis that species-specific molecular markers are superior to universal markers for detecting individual species at low abundance.

In the single-locus PCR experiment, primers Tila1, Tila2, Tila3, and Tila4 all achieved a 95% detection rate at a DNA concentration of 0.0023 ng/μL (Fig. 4).

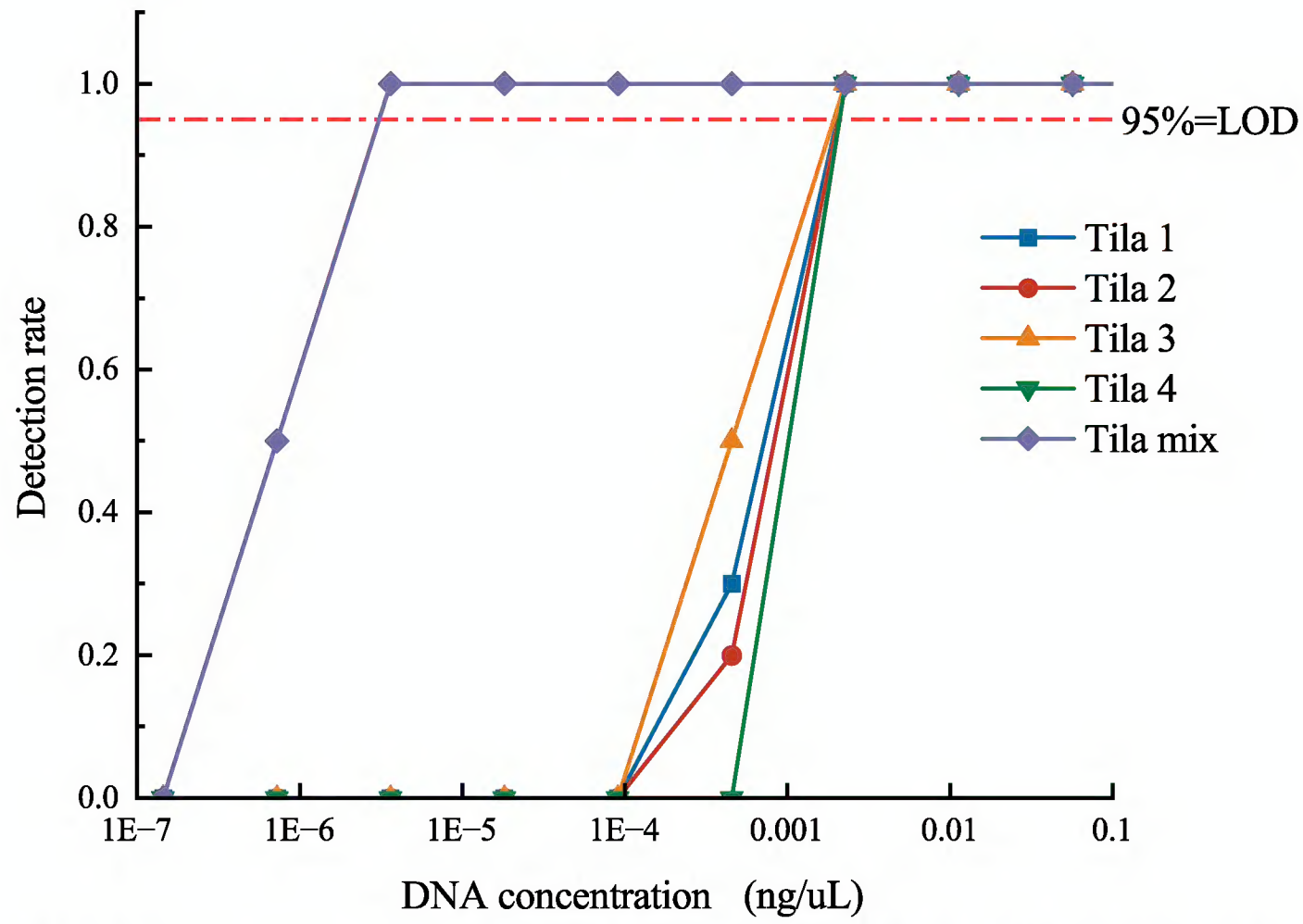

Figure 4. Detection rates of individual primer sets (Tila1, Tila2, Tila3, and Tila4) and the primer combination (Tila-mix) at different DNA concentrations (ng/μL). This figure illustrates the 95% limit of detection (LOD) and the consistency of detection at low template concentrations. Tila-mix refers to the combination of all four primer pairs.

This result indicates that at a concentration of 0.0023 ng/μL, each primer pair could effectively amplify the target DNA, demonstrating high detection sensitivity. The detection sensitivity was significantly enhanced when all four primers were combined in a multiplex PCR experiment. At an extremely low DNA concentration of 2.66×10^{-6} ng/μL, the primer combination reached a 95% detection rate, clearly superior to using any single primer alone (Fig. 4). These results showed that the multi-locus primer combination is significantly more effective than single primers in enhancing PCR detection sensitivity. This performance improvement confirms the effectiveness of the multi-locus eDNA strategy for detecting DNA at extremely low concentrations.

During the multiplex PCR experiment, we observed that even at extremely low DNA concentrations (4.54×10^{-4} ng/μL), both Tila1 and Tila4 primers amplified tilapia DNA successfully. In contrast, Tila3 primers amplified at even lower concentrations (3.63×10^{-6} ng/μL), while Tila2 primers only amplified at a concentration of 2.3×10^{-3} ng/μL. Among the four primers, Tila3 demonstrated the highest amplification efficiency in low-concentration DNA samples.

To better simulate the complex background of natural aquatic environments, we established an experimental system in which genomic DNA from 20 common freshwater fish species (as described in the "primer validation" section) was kept constant as background, while tilapia DNA was serially diluted to mimic low-abundance conditions. When combining the advantages of taxon-specific primers and multi-locus eDNA markers, we found that the performance of multi-locus and taxon-specific methods was much better than using a single metabarcoding marker, tele02. When the tilapia DNA concentration was 0.00833 ng/μL, the proportion of target reads amplified using tele02 dropped to 1.2% of the total sequencing reads, with the remaining 98.8% primarily corresponding to

background freshwater fish species. However, at the same concentration, Tila-mix amplification still yielded 40.13% of reads corresponding to tilapia, while the remaining 59.87% of reads were likely primer dimers or low-complexity sequences that could not be reliably annotated. At a lower concentration of 6.66×10^{-5} ng/μL, Tila-mix amplification maintained a proportion of target reads above 8.7%, whereas with tele02, the proportion had already approached zero. Detailed taxonomic composition of non-target reads is provided in Table 3.

Table 3. Proportional composition of sequencing reads from amplification products using Tila-mix and tele02 primers under different DNA concentrations.

Primer	The DNA concentration of tilapia (ng/μL)	Target tilapia	Background fish	Unannotated / primer-dimer reads
Tila-mix	8.33×10^{-3}	40.13%	0%	59.97%
	6.66×10^{-5}	8.70%	0%	91.30%
Tele02	8.33×10^{-3}	1.20%	96.40%	2.40%
	6.66×10^{-5}	0%	98%	2%

Preliminary survey of tilapia invasion in China

Tilapia were successfully detected at seven of the nine sampling sites, including the Xin'an River, the Dongjiang River, the Minjiang River, the Yangtze River, the Ganjiang River, the Shenzhenhe River, and the Xijiang River (Fig. 5). It should be noted that due to the close phylogenetic relationship between *S. galilaeus* and *O. aureus*, primers Tila1 and Tila3 could not distinguish them. However, primers Tila2 and Tila4 effectively differentiated these two species and thus can be used for their identification. Multiple tilapia species were detected at several sites in the Xin'an, Dongjiang, Shenzhenhe, and Xijiang rivers, with *C. zillii* and *O. niloticus* being the most common. In contrast, tilapia were detected at only one of the three sampling sites in each of the Minjiang River and the Ganjiang River. Overall, the sampling points in the Dongjiang River and the Shenzhenhe River exhibited higher diversity, with *C. zillii* and *O. niloticus* being the most common species.

Besides the Tila markers, we also applied tele02 to amplify the field samples. The results showed that at all sites, the Tila markers retrieved a higher proportion of tilapia sequences than those obtained using the tele02 markers. Tilapia were identified in 17 samples (seven rivers) using Tila-mix but only confirmed in 10 samples (5 rivers) using tele02. Furthermore, in all samples, Tila-mix retrieved the same or more tilapia species than tele02.

Discussion

Enhanced detection efficiency of tilapia using a multi-locus, taxon-specific multiplex PCR assay

Our results demonstrate that the newly designed primer combination (Tila-mix) significantly outperforms the widely used universal tele02 primers in both laboratory and field conditions, particularly when target DNA is present at low concentrations. This improvement is crucial for monitoring invasive tilapia in natural environments, where DNA is often degraded or present in trace amounts. To better understand the performance of the multiplex assay, we also compared the relative

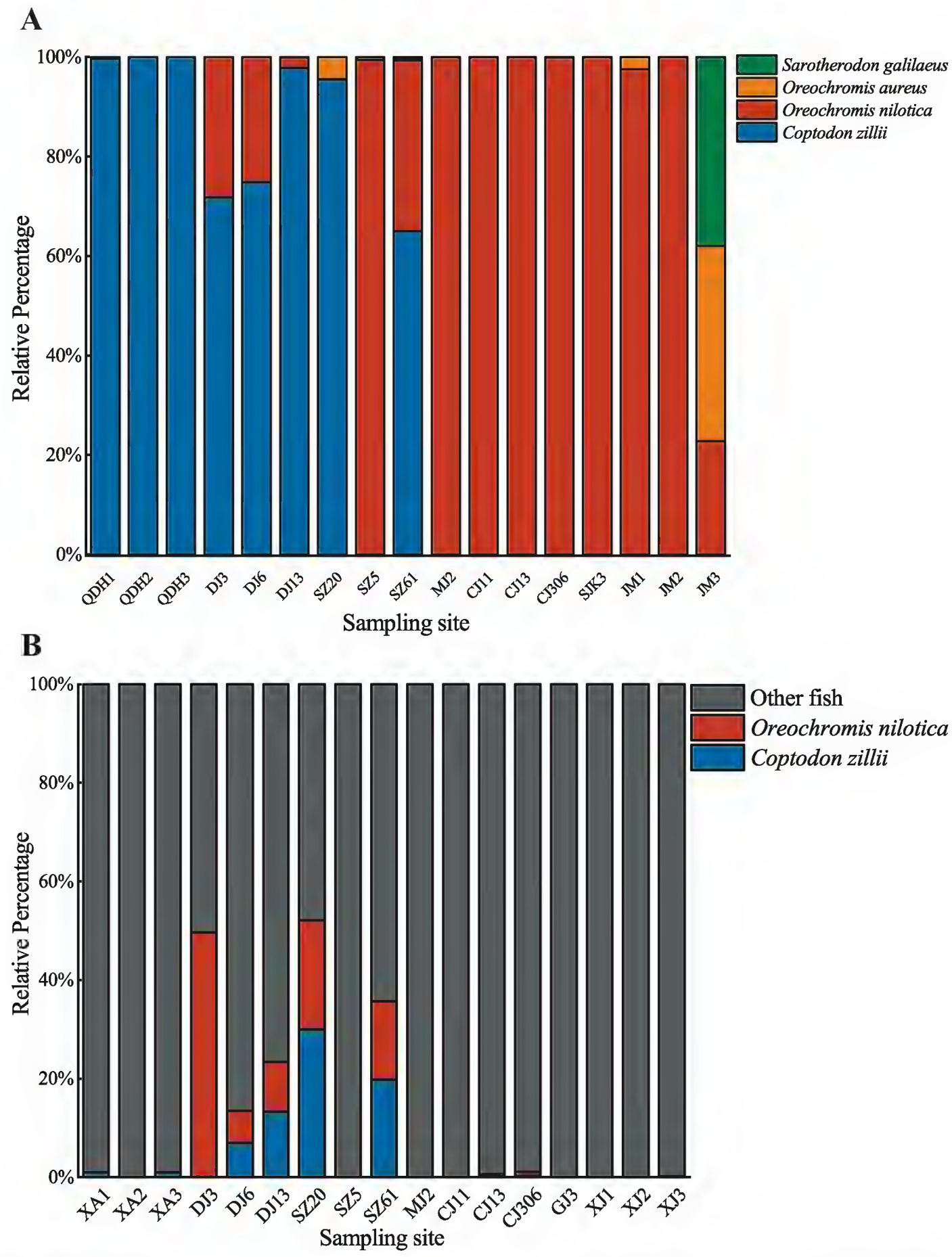

Figure 5. Percentage of tilapia species composition at different sampling sites. **A.** Results based on the Tila Mix primers; **B.** Results based on the tele02 universal primers. XA: the Xin'an River; DJ: the Dongjiang River; SZ: the Shenzhenhe River; MJ: the Minjiang River; CJ: the Yangtze River; GJ: the Ganjiang River; XJ: the Xijiang River.

amplification efficiency of the individual primers within the Tila-mix. In multiplex PCR assays combining all four primer sets, Tila3 demonstrated the highest amplification efficiency, successfully amplifying DNA even at the lowest tested concentrations (3.63×10^{-6} ng/μL), whereas Tila2 only amplified at concentrations above 2.3×10^{-3} ng/μL. This discrepancy can be explained by differences in amplicon length: Tila3 targets the shortest fragment, making it easier to amplify under competitive multiplex conditions, while Tila2 targets the longest region, which likely reduces its efficiency. Notably, at low DNA concentrations, Tila-mix retained a high proportion of target reads, while tele02 failed to detect targets effectively (Table 3). This is likely due to the broad binding capacity of universal primers, which can amplify non-target DNA and dilute the representation of the intended

target. In contrast, species-specific primers minimize off-target amplification and maintain performance in complex samples.

Furthermore, our study highlights the value of targeting multiple loci to enhance detection reliability. eDNA-based studies often focus on optimizing sample collection and DNA extraction (Goldberg et al. 2016), but amplification strategies also play a key role in detection success. eDNA is typically fragmented and degraded (Furlan et al. 2016), and single-locus assays may fail if the target region is damaged or poorly conserved. Prior research has shown that multi-locus strategies can increase robustness by compensating for dropout at any single site (Farrington et al. 2015; Brys et al. 2023). In this study, the term "multi-locus" refers to multiple mitochondrial gene regions (16S, ND1, COI, and D-loop), all of which are part of the multicopy mitochondrial genome. Targeting distinct loci enhances amplification robustness, particularly when some regions are degraded or inhibited. We acknowledge the potential presence of NUMTs – non-functional mitochondrial gene copies transferred to the nuclear genome – that may retain primer-binding sites. However, no NUMT interference was observed in our study: all amplified sequences showed high identity to mitochondrial references, and no anomalous nuclear-like variants were detected in the sequencing data. In our field trials, detection success varied across sites and primers, yet combining multiple markers improved the overall detection rate and reduced false negatives. For instance, the Xin'an and Dongjiang rivers exhibited 100% detection success with all four primers, while at other sites, detection success varied between 25% and 75%.

Preliminary results on the current status of tilapia invasion in China

Tilapia have become widely naturalized in southern Chinese freshwater systems, with natural populations recorded in river systems south of the Yangtze River, including the Pearl River Basin, Hainan Island, and the coastal rivers of Guangdong, Guangxi, and Fujian provinces (Gu et al. 2018). Among the introduced tilapia species in China, *O. niloticus* and *C. zillii* are the most commonly established in natural waters. *Oreochromis niloticus* typically dominates warmer southern habitats due to its larger size, stronger competitiveness, and pollution tolerance, while *C. zillii*, with its greater cold tolerance, has expanded into relatively northern regions such as Hunan, Jiangxi, Hubei, and Sichuan provinces (Gu et al. 2018).

In this study, we detected tilapia at several sampling points, including the Xin'an, Yuan, Dongjiang, Shenzhenhe, Minjiang, Yangtze, Ganjiang, Xijiang, and Yihe rivers. *C. zillii* was detected in the Xin'an River, consistent with reports of its expansion into this area (Liu et al. 2024). Additionally, we detected *O. aureus* and *S. galilaeus* at a small number of sites, suggesting that while the presence of these species in the Xin'an River has not been widely reported, our study revealed their limited presence in the area. *C. zillii* dominated in the Xin'an River, likely due to its cold tolerance and the region's relatively mild winters. In contrast, *O. niloticus* was more abundant in southern coastal rivers such as the Dongjiang and Shenzhenhe, where warmer temperatures and richer food resources provide favorable conditions for its growth. At the northern edge of our study region, tilapia were not detected in the Yihe River, which represents the northernmost site sampled. Despite its connection to the Yangtze River, this result suggests that the Yangtze may still be the front and a barrier to tilapia invasion, probably due to low winter temperatures in this region. Similarly, tilapia were absent in

the Yuan River, contrary to reports of their expansion in Hunan Province (Pearl River Fisheries Research Institute 2022). This discrepancy may reflect unsuitable microhabitats at our sampling site or the absence of stable natural populations in this particular region.

Because the sampling sites in this study were determined mainly by existing environmental monitoring programs, they did not fully account for the habitat preferences of tilapia. As a result, some sites may not have been optimal for detecting this species. Future monitoring efforts should include more targeted sampling sites to better track tilapia invasions and invasion fronts in China. Another limitation of this study is that tilapia hybridization, although common in aquaculture and rare in natural waters, cannot be resolved with our mitochondrial markers, which only capture the maternal lineage. Future work could integrate nuclear markers to distinguish hybrids and provide a more comprehensive understanding of tilapia invasion dynamics.

Conclusion

We successfully developed multiple specific primers for tilapia and conducted preliminary monitoring of tilapia invasion in China using eDNA technology and multiplex PCR analysis. The results showed that, compared to traditional universal primers and single-locus methods, taxon-specific primers and multi-locus approaches have higher detection sensitivity in low-concentration target samples. Tilapia were found to be widely distributed in areas including the Xin'an River, the Dongjiang River, the Shenzhenhe River, the Minjiang River, the Yangtze River, the Ganjiang River, and the Xijiang River, further verifying the effectiveness and reliability of the developed methods. The specific primer combinations demonstrated excellent performance in improving detection sensitivity and lowering detection limits, especially in complex environmental samples. The findings of this study provide a new approach for the early detection and monitoring of tilapia invasion, which is important for the management and protection of aquatic ecosystems.

This study developed multiple tilapia-specific markers and multiplex PCR protocols for tilapia detection. The strategy used to develop these protocols may inspire similar practices for monitoring other invasive species. All data from the study are freely accessible at https://doi.org/10.6084/m9.figshare.27643779.

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Additional information

Conflict of interest

The authors declare the following: Chenhong Li holds a patent (Patent Number: CN202411112362X) related to [A primer set and kit for detecting invasive species tilapia in water bodies based on environmental DNA]. There is no financial or commercial interest associated with this patent in relation to the research described in this article.

Ethical statement

No ethical statement was reported.

Use of AI

No use of AI was reported.

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Author contributions

M.F., Y.Q., and C.L. designed the study. M.F. and Y.Q. completed laboratory work. M.F. and H.W. analyzed the data and interpreted the results. M.F. wrote the manuscript. All authors contributed to revising the manuscript and approved it for publication.

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Data availability

ASV sequences generated by PCR amplification with taxon-specific primers at various sampling sites, as well as ASV sequences obtained from tilapia DNA at different concentrations using taxon-specific primers, have been deposited at https://doi.org/10.6084/m9.figshare.27643779. The dataset includes detected tilapia species and corresponding reads across different sampling sites and primer combinations, ensuring comprehensive accessibility for further analysis and verification.

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Supplementary material 1

Supplementary figure

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Data type: png

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